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Development, validation and application of a novel HPLC-MS/MS method for the measurement of minocycline in human plasma and urine

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ABSTRACT

New treatments are urgently required to treat infections caused by multi-drug resistant *Acinetobacter baumanni,*. To address this need, a new formulation of Minocin®, (minocycline for injection) has been developed that allows for higher doses of minocycline to be administered. Phase 1 clinical trials were conducted in healthy volunteers to assess the safety and pharmacokinetics (PK) of this new formulation at higher doses. In order to generate PK data, novel, selective and simple HPLC-MS/MS based assays were developed and validated for the determination of minocycline (MC) in human plasma and urine. The respective working ranges were 0.05 to 30 mg/L and 0.1 to 30 mg/L. Removal of endogenous proteins with trichloroacetic acid was used as a simple means of extracting MC from the samples. An analogue, tetracycline was used as the internal standard (IS). Chromatographic separation, including that of MC from its 4-epimer (4-EMC), was achieved on a Waters XBridge BEH C18 column (50 x 4.6 mm ID, 5 μm) with gradient elution. The mobile phases comprised water containing 5 mM ammonium formate at a pH of 2.5, and methanol containing 5 mM ammonium formate. The internal standard (IS) was tetracycline, a structural analogue of minocycline.

The methods were fully validated and met regulatory acceptance criteria for intra-run and inter-run accuracy and precision, carryover, dilution integrity and matrix effects. Mean extraction recoveries ranged between 64.3% and 84.6% for MC and 64.3% for the IS. There was no significant ion suppression or enhancement for MC or the IS. The validated assays were successfully applied to 1423 plasma and 689 urine samples from a Phase 1 clinical study.

There was no evidence of instability, or significant interconversion between MC and 4-EMC, in stored clinical samples, spiked plasma and urine samples, or their extracts, under various test conditions.

1. Introduction

Tetracyclines (TCs) are important broad spectrum antibiotics which are active against gram-positive and gram-negative bacteria. They were first used in clinical practice in the 1950′s. Minocycline (MC, Fig. 1) is a second generation semi-synthetic tetracycline that was first described in the 1960s and became commercially available in 1971 [1–3] MC, reportedly the most lipophilic tetracycline, is absorbed rapidly, readily crosses the blood–brain barrier [4–6] and has potential for treating a wide range of non-infectious illnesses [7].

MC has been proposed as a treatment for infections caused by increasingly multi-drug resistant (MDR) *Acinetobacter baumanni* (AB) and methicillin-resistant *Staphylococcus aureus* (MRSA) [8,9]. The risk of MDR AB to public health has been highlighted by the US government [10,11] and the European Commission [12]. MC is active against strains that are resistant to newer TCs such as doxy-

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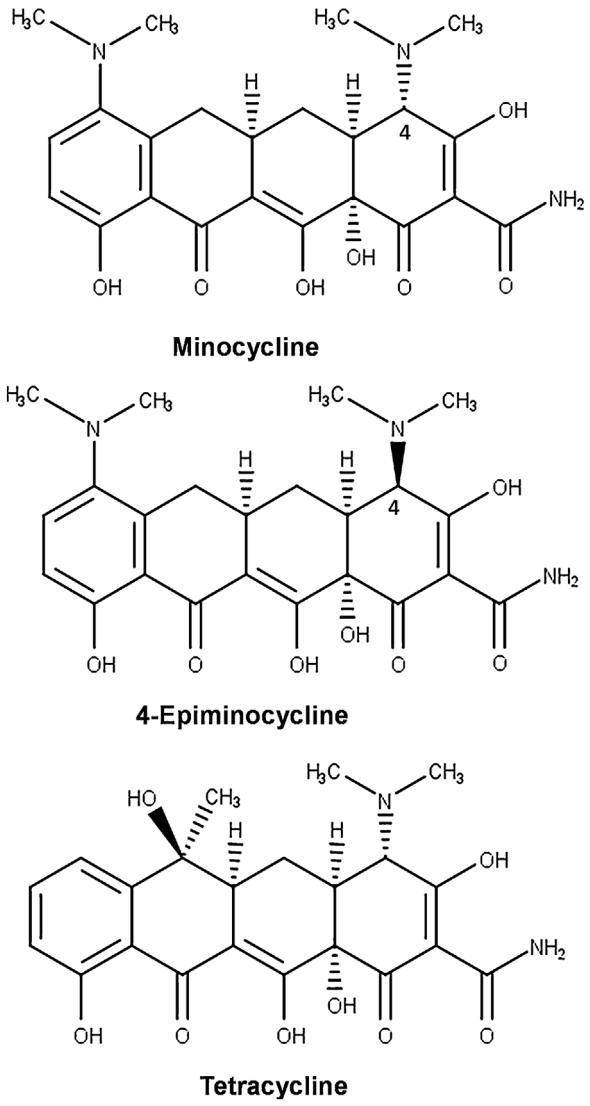

Fig. 1. Structures of Minocycline, 4-Epiminocycline and Tetracycline.

cycline and tigecycline [13]. Intravenous (IV) formulations of MC have been reviewed for their effectiveness in treating MDR AB [14]. To address this need, a new formulation of Minocin®, (minocycline for injection) has been developed that allows for higher doses of minocycline to be administered. [15]. Oral and IV formulations, with a maximum dose of 400 mg (200 mg twice daily), have been approved in the US for several decades but only oral doses have been approved for use in the countries of the EU. A Phase 1 clinical study, was performed to investigate the safety, tolerability and pharmacokinetics (PK) of single and multiple doses of Minocin®, in healthy adults [15]. This study included doses higher than those approved in the US. MC was developed prior to the widespread use of Pharmacokinetics-Pharmacodynamics (PK-PD) modelling when clinical doses were not systematically evaluated or optimised [16].

Bioanalytical methods, based on HPLC-MS/MS, for the determination of MC in plasma and urine were developed to support this study. Urine analysis was needed to confirm the minor role of renal clearance at higher doses [17].

Numerous methods based on HPLC and HPLC-MS/MS have been reported for measuring MC in a wide variety of matrices, as reviewed thoroughly by Patel et al [18]. However, to our knowledge, only two publications have reported the use of HPLC-MS/MS

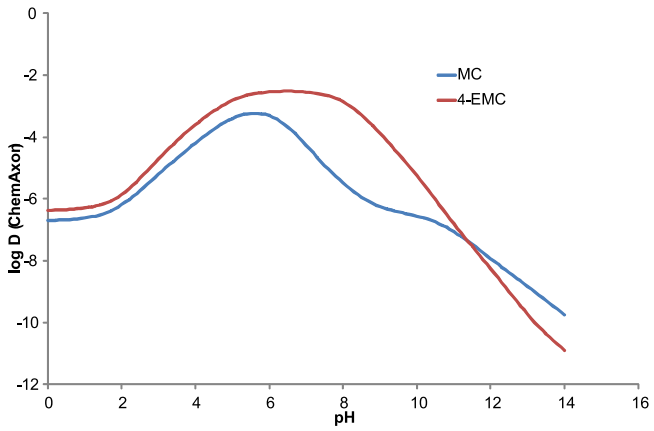

Fig. 2. Calculated Log D vs pH for MC and 4-EMC.

for the analysis of MC in human plasma [18,19] and none for urine. Araujo et al [19] describe a limited validation.

MC, like other TCs [20–22], readily epimerises at the 4-position to 4epiminocycline (4-EMC, Fig. 1) *in vitro* and *in vivo* [23]. According to Nelis et al [24] this could happen in the bladder. Epimers of TCs are reported to have different antibacterial and toxicological properties [22,25,26]. They pose a significant challenge for selective bioanalysis, being isobaric with the parent drug and having very similar physicochemical properties (Fig. 2). Insufficient chromatographic resolution leads to overestimates of the levels of parent drug, especially at later time points after dosing. Patel et al [18] describe the only reported method that uses HPLC-MS/MS to analyse MC in human plasma whilst separating 4-EMC.

Trichloroacetic acid (TCA) was used to extract MC from human plasma and urine samples in the assays described herein. A structural analogue, tetracycline (Fig. 1) was used as the internal standard (IS).

2. Materials and methods

2.1. Chemicals and reagents

The following chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA): minocycline hydrochloride, tetracycline, ammonium formate and trichloroacetic acid (TCA). 4-EMC was obtained from Santa Cruz Biotechnology (Dallas, Texas, USA). Water, methanol and formic acid were obtained from Fisher Scientific (Loughborough, UK). Dimethyl sulfoxide (DMSO) was obtained from VWR International (Radnor, PA, USA). Non-sterile, citrated dialysed human plasma with K2 ethylenediaminetetraacetic acid (EDTA) was purchased from TCS biosciences (Botolph Claydon, UK). Urine was provided by volunteers in-house. All reagents were HPLC-grade or its equivalent, at the minimum.

2.2. Preparation of solutions, calibration standards, and quality controls

All re-usable glassware was washed with water containing 0.1% v/v formic acid, water and methanol, then tested for contamination prior to use. Stock solutions of MC and IS were prepared in aqueous methanol, that of 4-EMC in DMSO. Intermediate and working solutions were prepared by dilution with water. Calibration standards were prepared in plasma at the following concentrations: 0.05 (Lower limit of quantification, LLOQ), 0.1 (2 x LLOQ), 0.5, 1.5, 3.0, 5.0, 7.5, 15, 25 and 30 mg/L. Calibration standards were prepared in urine at the following concentrations: 0.1 (LLOQ), 0.2 (2 x LLOQ), 0.5, 1.5, 3.0, 5.0, 7.5, 15, 25 and 30 mg/L. Low, medium and high QCs were prepared at concentrations of 0.15, 7 and 24 and 0.3,

Table 1
LC gradient.

Time (min)	Mobile phase (%)		Flow rate (mL/min)
	A	B	
0	90	10	0.8
0.5	90	10	0.8
5.5	60	40	0.8
5.6	0	100	0.8
5.7	0	100	1.5
6.1	0	100	1.5
6.2	90	10	1.5
7.2	90	10	1.5
7.4	90	10	0.8

Mobile phase A: water containing 5 mM ammonium formate (pH of 2.5).
Mobile phase B: water-methanol, 5/95 v/v containing 5 mM ammonium formate.

7 and 24 mg/L in plasma and urine respectively. For the purposes of the validations, additional QCs were prepared at the following concentrations: LLOQ and 2 x LLOQ. All solutions and test samples were stored at ca. -80 °C and allowed to reach room temperature on the day of analysis. On each day of analysis, a final IS working solution was prepared at a concentration of 1 mg/L in water containing 10% w/v TCA.

2.3. Extraction of plasma and urine samples

100 μL of sample was dispensed into a 0.5 mL polypropylene Eppendorf® tube. The sample was then mixed thoroughly with 100 μL of IS working solution. After being left to stand at ca. 4 °C for 10 min, the sample was centrifuged for 5 min at 17,000 *g*. 100 μL of supernatant was then transferred to a glass HPLC vial containing 900 μL of water. The final extract was thoroughly mixed prior to analysis. Full ranges of calibration standards were injected at the start and end of each analytical run. Zero and blank samples, with and without IS respectively, were included with each set of standards.

2.4. HPLC-MS/MS conditions

A Shimadzu Prominence® (Kyoto, Japan) LC system was coupled to a Sciex triple quadrupole 4000 QTRAP mass spectrometer (AB Sciex, Warrington, UK). Sciex Analyst v.1.5.1 software was used to control the HPLC-MS/MS system and collect the MS data. High purity nitrogen was supplied to the MS by a Thyster-TF/1 generator (F-DGSi, Every, France). The LC system consisted of a SIL20AC autosampler, two LC-20AD pumps, a CTO-20 AC column oven and a CBM-20A controller. The autosampler was set a temperature of 4 °C and programmed to inject 5 μL of each sample. Gradient separation (Table 1) was achieved, at a temperature of 40 °C, on a Waters (Milford, Massachusetts, USA) XBridge BEH C18 column (5 μm, 130 Å, 50 x 4.6 mm ID, part no. 186003113) protected with an Upchurch (Hichrom, Reading, UK) precolumn filter unit fitted with a 0.5 μm frit. Each LC injection cycle was 7.5 min long. A 6-port, 2-way, Valco valve (VICI AG, Schenkon, Switzerland) was set to divert eluate containing the peaks of interest to the MS from 3.5 to 5.5 min.

Mobile phase A comprised water containing 5 mM ammonium formate, diluted from a 100 mM stock solution buffered to a pH of 2.5. Mobile phase B comprised water-methanol, 5/95 v/v, and 5 mM ammonium formate. The autosampler wash contained water-methanol, 50/50 v/v and was programmed to wash the injection needle before and after injection.

Multiple Reaction Monitoring (MRM) data were collected by the MS using a Turbo V™ source in electrospray ionisation (ESI) positive mode. The source temperature was set at 600 °C, ion spray voltage at 5500 V, ion source gases 1 and 2 at 30 and 45, curtain gas (CUR) at 40 and collision gas (CAD) at 7. The MRM transitions for MC/4EMC and IS were set to *m/z* 458.4→441.2 and 445.3→410.3 respectively, with a dwell time of 150 ms. MS parameters were optimised by infusion of the analyte and IS into the HPLC-MS/MS system.

2.5. Data analysis

Raw data were processed by Analyst v.1.5.1 using the ratio of the peak areas of MC to IS.

2.6. System suitability test solutions

The general performance and chromatographic resolution of the HPLC-MS/MS system were evaluated at the start of each run by injections of separate solutions of MC and IS, MC and 4-EMC, in water containing 0.5% w/v TCA.

2.7. Validation of the assays

The assays in plasma and urine were validated following the guidelines issued by the US Food and Drug Administration and European Medicines Agency [27,28]. The following properties were assessed: calibration model, accuracy and precision, selectivity, carryover, matrix effects, dilution integrity, stability, ionisation effects and extraction recoveries.

2.7.1. Calibration model

Calibration lines were constructed from non-zero standards using a linear regression model with $1/x^2$ weighting [29].

2.7.2. Acceptance of runs

The calibration was deemed acceptable when ≥66.7% of the non-zero standards were within ≤15% of the nominal concentration; within ≤ 20% at the LLOQ. Results from runs containing clinical samples were accepted when ≥66.7% of QC and at least one replicate at each QC level were within ≤15% of the nominal concentration. Results from diluted samples were accepted when ≥66.7% of dilution QCs were within ≤15% of the nominal concentration. In the clinical study, concentrations below the LLOQ were defined as below the LLOQ (BLQ). Those above the ULOQ were reanalysed after dilution.

2.7.3. Selectivity

Six individual samples of blank matrix were analysed. For plasma, one lipaemic and one haemolysed sample were included. Acceptable limits for selectivity were defined as by the response being ≤ 20% of the LLOQ for MC and ≤ 5% for the IS, as based on the average response for the run.

2.7.4. Carryover

After the highest calibration (ULOQ) standard, three blank samples were injected. Acceptable carryover was defined as the detector response for MC being ≤ 20% of the LLOQ and that for the IS being ≤ 5% of the average for the IS throughout the run.

2.7.5. Accuracy, precision and LLOQ

Intra-run accuracy and precision was determined from the mean values from six replicates of each QC. Inter-run batch accuracy and precision were determined using the mean values from duplicate analyses at each QC concentration over eleven and nine runs for plasma and urine respectively. Precision was regarded as acceptable if the coefficient of variation (CV) was ≤ 15% for each QC and ≤20% at the LLOQ. Accuracy was regarded as acceptable if within ±15% of the nominal concentration for each QC and within ±20%

Table 2
Ionisation effects and extraction recoveries.

Deproteinising agent[1]	QC	Plasma[2]		Urine[2]	
		Extraction recovery (%)	Ionisation effect (%)	Extraction recovery (%)	Ionisation effect (%)
Trichloroacetic acid (TCA)[3]	Low	64.3	109	115	111
	Medium	84.6	112	119	98.3
	High	77.8	110	112	102
	IS	64.3	109	109	94.4
Acetonitrile (MeCN)	Low	64.0	61.4	66.2	79.9
	Medium	83.8	58.7	52.0	70.0
	High	66.9	68.4	55.1	64.1
	IS	53.0	115	35.9	141
Methanol (MeOH)	Low	73.1	61.7	48.8	51.9
	Medium	83.5	65.7	54.5	48.6
	High	84.8	54.7	59.7	61.7
	IS	55.8	99.0	44.9	95.7

[1] Volumes of 1:1, 2:1, 4:1 for TCA, MeCN and MeOH to plasma/urine respectively, followed by 10-fold dilution with water.
[2] Data obtained from mean values of 6 replicates for TCA; 4 for MeCN and MeOH.
[3] Met acceptance criteria of CV(%)s being < = 15%.

at the LLOQ. At the LLOQ, the detector response was required to be at least five times that obtained using a blank sample.

2.7.6. Matrix effects

Matrix effects were assessed using the method reported by Matuszewski et al [30]. Six batches of blank plasma and urine were analysed after being spiked with MC at the same nominal concentrations as the low and high QCs. These samples were extracted alongside aqueous reference solutions containing the same concentrations as the low and high QCs. All samples were analysed in triplicate. A CV of $\leq$ 15% for the IS-normalised matrix factor (Table 2) was deemed acceptable.

2.7.7. Dilution integrity

Blank plasma and urine samples were prepared at concentrations of twice and ten times the ULOQ. These were diluted 10-fold and 50-fold, respectively, with blank matrix prior to extraction. Dilution integrity was deemed acceptable if mean accuracy was within ±15% of the nominal concentration and the CV was $\leq$ 15% for six replicates.

2.7.8. Stability

The stability of MC in plasma and urine was determined using low and high QCs under the following storage conditions: room temperature, both protected from and exposed to light, refrigerated at ca. 4 °C, frozen at ca. -20 °C and ca. -80 °C and for up to six freeze/thaw cycles. Stability of extracts was assessed after refrigeration at ca. 4 °C, undiluted and diluted, and in the autosampler tray set to 4 °C. Freshly extracted samples were used as comparators. Mean baseline and nominal values were used to assess stability. Stock and working solutions of MC and IS were assessed after storage at ca.-80 °C by comparison with freshly prepared solutions.

2.7.9. Ionisation effects and extraction recovery

Ionisation effects and extraction recoveries were determined using blank matrix samples spiked after extraction. The concentrations in the final extracts were set to be equivalent to 100% extraction recovery for the low, medium and high QCs, and the IS. Ionisation effects were assessed by comparing the responses with those obtained from matrix-free solutions at the same QC levels. Extraction recoveries were assessed by comparing the responses of the samples spiked after extraction with those from the corresponding QCs. The mean values from six replicates for each sample were used for the assessment. A CV of $\leq$ 15% for the recoveries at each QC level was deemed acceptable with one QC level being used for assessing the IS.

2.8. Application to clinical samples

The fully validated methods for MC in plasma and urine were applied to samples collected during the clinical study described earlier. Blood samples were collected in EDTA-containing vacutainers [18,19]. These were centrifuged at 2000 *g* for 10 min at 4 °C then immediately transferred to storage at ca. -80 °C. Test samples were transported frozen, on dry ice, for analysis at Southmead Hospital, Bristol, UK. Two replicates of the low, medium and high QCs were interspersed throughout each analytical run.

2.9. Incurred sample reanalysis (ISR)

A minimum of 5% of plasma and urine samples from the safety and tolerability study were reanalysed. Results from the reanalyses were compared to the original values. ISR was deemed acceptable when the percentage difference between the pairs of results was < = 20% for > = 66.7% of the samples.

3. Results & discussion

3.1. Optimisation of the chromatographic conditions and robustness of the HPLC-MS/MS system

In the absence of an ion-pairing agent, adequate separation of MC from 4-EMC with an acceptable chromatographic peak shape could not be obtained with a mobile phase containing acetonitrile on the following range of stationary phases, packed in 50 x 2.1 mm ID columns: 2.7 μm Halo™ C18, 2.6 μm Kinetex™ XB-C18, 2.6 μm Accucore™ AQ and 2.6 μm Accucore™ Phenyl-Hexyl. Methanol, combined with 5 mM aqueous ammonium formate on a XBridge BEH C18 stationary phase gave the best compromise between the resolution of MC from 4-EMC and the injection cycle time, on a conventional LC system. Reducing the pH to 2 removed the resolution whilst increasing the pH to 3 led to improved resolution but significantly longer retention times. Unlike reported elsewhere [18], the assays described here avoid the use of an ion pairing agent but use the following: a wash stage for the column with each injection cycle, diversion of eluate from the MS and a pre-column filter. In our view, this approach increases the lifetimes of the columns, reduces contamination of the LC system and avoids time-consuming, potentially costly, additional maintenance of the MS. This was demonstrated by the robustness of the assays when applied to clinical samples.

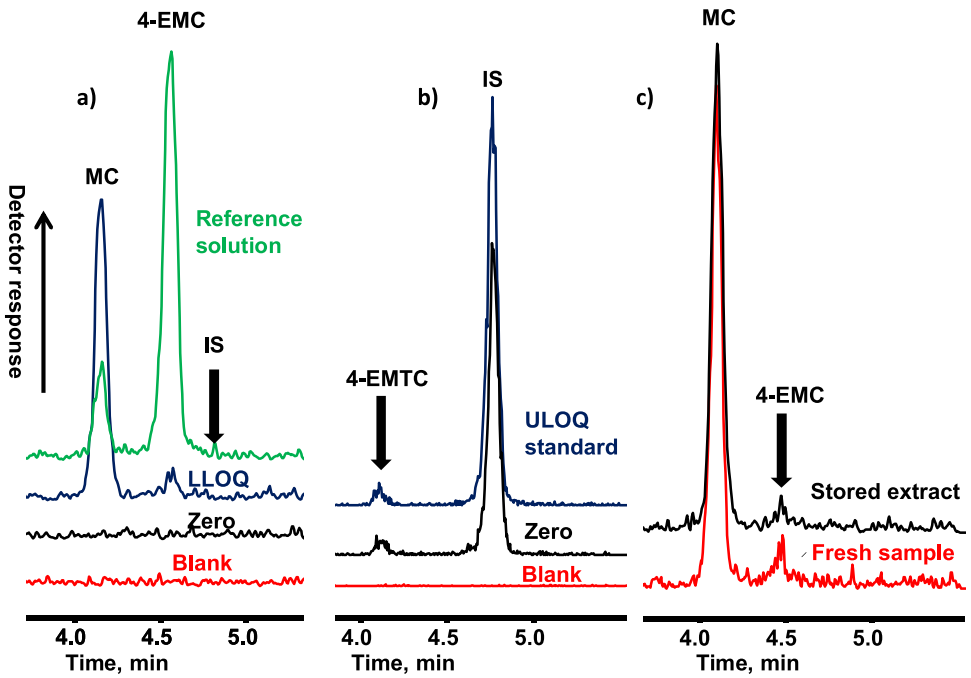

Fig. 3. Representative chromatograms from plasma validation samples.

Table 3
Stability of Minocycline in plasma and urine.

Test/temperature	Time/cycle	QC	Plasma[1]		Urine[1]	
			Meanaccuracy(%)	CV (%)	Meanaccuracy(%)	CV (%)
RT/protected from light	0 (baseline)	Low	88.2	1.75	95.0	2.74
		High	92.9	2.05	94.3	1.02
	6 hours	Low	90.4	4.50	95.8	0.402
		High	88.9	4.36	95.6	3.09
RT/exposed to light	0 (baseline)	Low	88.2	1.75	98.0	6.78
		High	92.9	2.06	91.4	2.11
	6 hours	Low	97.3	4.17	94.6	2.83
		High	99.7	3.50	91.1	2.30
Freeze-thaw cycles[1]	0 (baseline)	Low	103	6.15	109	7.14
		High	100	8.57	103	2.43
	last cycle	Low	116[2]	7.62	103	3.56
		High	97.2	3.84	95.7	1.65
ca. 4 °C	0 (baseline)	Low	93.3	4.56	102	5.10
		High	94.6	2.58	91.5	3.03
	24 hours	Low	90.2	8.66	91.6	4.06
		High	94.3	1.35	88.9	3.90
ca. -80 °C	0 (baseline)	Low	99.8	3.01	107	2.12
		High	108	3.72	101	3.29
	6 Months	Low	96.2	4.72	107	1.57
		High	114	7.78	110	0.438

Accuracy of mean and CV (%) obtained from 3 individual replicates.
Abbreviations: RT = Room temperature.
2Accepted as within 15% of baseline value.
[1] 6 cycles for plasma and 5 cycles for urine.

3.2. Choice of extraction method and epimerisation

Chromatographic separation of MC from 4-EMC in a matrix-free reference solution is demonstrated in Fig. 3. TCA has not been the deproteinising agent of choice when extracting TCs from serum or plasma. Here, TCA gave higher sensitivity as the final extract was less dilute, extraction recoveries were similar and ion suppression was reduced, when compared to acetonitrile or methanol (Table 2). The concern that TCA could catalyse epimerisation was shown to be unfounded by the results of the investigations into the stability of MC and the IS in stored extracts (Tables 3 and 4, Figs. 3 and 4). As this applied to both urine and EDTA-plasma, it is theorised that the ion pairing properties of TCA are mainly responsible.

3.3. Ionisation effects, extraction recoveries and choice of IS

The similar ionisation properties and extraction recoveries of the IS (Table 2) compared to MC, its stability in sample extracts (Table 3) and the robustness of the assays supported the use of a structural analogue, tetracycline, as an IS. This was a cheaper and more accessible alternative to deuterated minocycline. Ionisation effects and extraction recoveries met the acceptance criteria.

3.4. Method validations

The assays described here met all the validation criteria outlined earlier with respect to the calibration model, selectivity, carryover,

Table 4
Stability of Minocycline and IS in plasma and urine extracts at ca. 4 °C.

Test conditions	Time	QC	Accuracy of mean (%)	
			Plasma	Urine
Autosampler tray	0 (baseline)	LLOQ	89.9	115
		2xLLOQ	92.2	99.0
		Low	93.7	102
		Medium	99.0	104
		High	96.0	98.9
	7 days	LLOQ	88.7	101
		2xLLOQ	92.0	101
		Low	90.3	102
		Medium	97.5	107
		High	96.0	101
Refrigerated	0 (baseline)	LLOQ	106	115
		2xLLOQ	96.0	113
		Low	99.8	102
		Medium	92.7	104
		High	108	98.9
	5 days (plasma) 7 days (urine)	LLOQ	106	111
		2xLLOQ	96.0	103
		Low	99.8	102
		Medium	92.7	95.0
		High	108	89.9
Refrigerated (undiluted)	0 (baseline)	LLOQ	106	115
		2xLLOQ	96.0	113
		Low	95.6	102
		Medium	92.7	104
		High	93.6	98.9
	5 days	LLOQ	88.4	98.7
		2xLLOQ	86.5	93.0
		Low	95.1	99.4
		Medium	103	105
		High	94.2	95.3

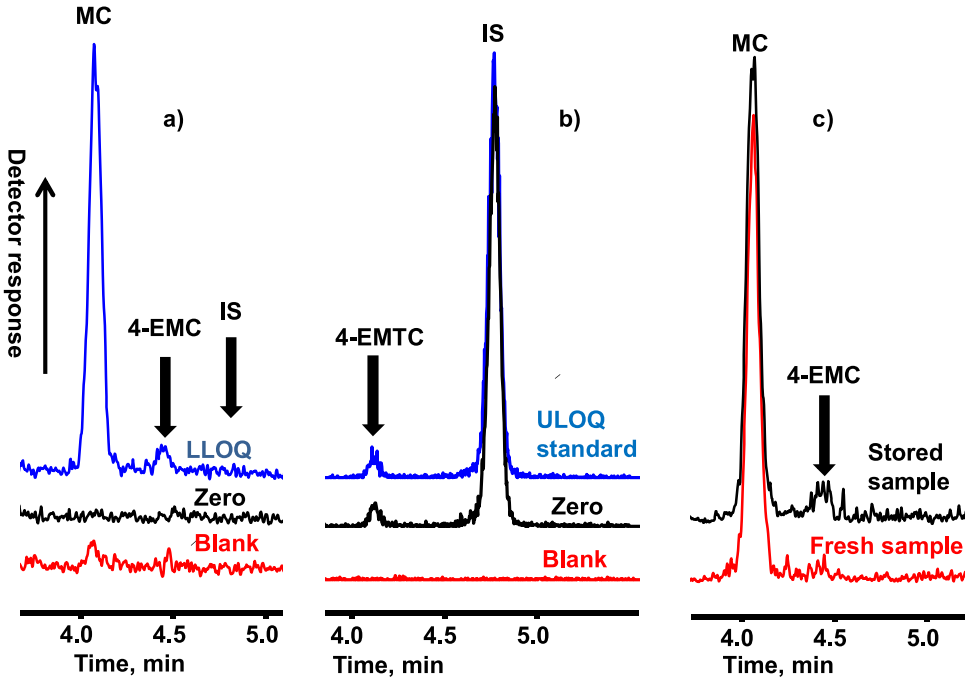

Fig. 4. Chromatograms from urine validation samples.

accuracy, precision, matrix effects and dilution integrity (Table 5). The validated ranges were 0.05–30 mg/L for human plasma and 0.1–30 mg/L for human urine. Peaks for MC and IS were not detected in the carryover test samples nor in the 6 blank plasma samples evaluated for matrix effects. MC was detected in 2 of the 36 blank urine replicates tested for matrix effects but the response was < = 20% of the LLOQ calibration standard.

3.5. Stability of MC in plasma and urine samples

MC was stable in plasma at room temperature, protected from and exposed to light, for up to 6 h, refrigerated for 24 h, for up to six freeze-thaw cycles and after ca. six months at ca. -80 °C (Table 3). MC was stable in urine under the same conditions but was tested for only up to five freeze-thaw cycles Table 3).

Table 5
Summary of validation results.

Test	Property		Plasma		Urine	
		QC	Value	n	Value	n
Calibration Model	Correlation coefficient	–	0.997±0.000885	28	0.998±0.000803	26
	Failed calibrators	–	23 (3.87%)	594	8 (1.55%)	515
Dilution integrity	Accuracy (%)	60 mg/L	93.6	6	104	6
		300 mg/L	99.6	6	94.4	6
	Precision, CV (%)	60 mg/L	7.23	6	5.75	6
		300 mg/L	3.71	6	8.27	6
IS-normalised Matrix Effects**	Mean (%)	Low	1.32	6	1.02	6
		High	1.17	6	0.951	6
	Precision, CV (%)	Low	1.98	6	4.67	6
		High	1.96	6	4.04	6
Intra-run accuracy and precision	Accuracy (%)	LLOQ	116	6	106	6
		2xLLOQ	108	6	101	6
		Low	109	6	105	6
		Medium	105	6	103	6
		High	98.6	6	104	6
	Precision, CV (%)	LLOQ	7.68	6	4.39	6
		2xLLOQ	4.94	6	3.46	6
		Low	4.22	6	2.53	6
		Medium	2.99	6	4.51	6
		High	2.18	6	2.72	6
Inter-run accuracy and precision	Accuracy (%)	LLOQ	109	16	101	18
		2xLLOQ	103	22	98.6	18
		Low	97.3	18	100	18
		Medium	101	22	102	18
		High	96.4	20	98.1	18
	Precision, CV (%)	LLOQ	14.6	16	9.43	18
		2xLLOQ	13.0	22	5.50	18
		Low	8.88	18	7.88	18
		Medium	8.73	22	6.37	18
		High	5.64	20	5.36	18

n = number of replicates or individual calibrators.
Analyte matrix factor (%) = 100 x analyte peak area of plasma or urine sample/analyte peak area of aqueous solution. IS matrix factor (%) = 100 x IS peak area of plasma/urine sample/IS peak area of aqueous solution. For each sample, IS-normalised matrix factor = Analyte matrix factor /IS matrix factor. Mean and precision calculated from 3 replicates for each of 6 blank samples.

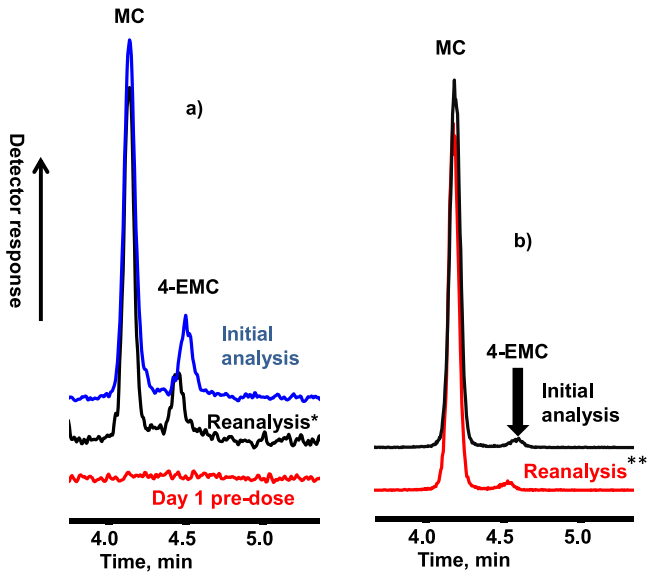

Fig. 5. Chromatograms from clinical plasma samples.

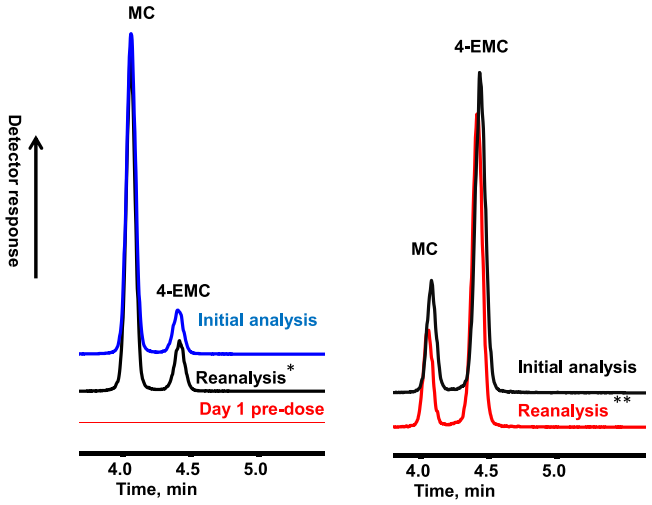

Fig. 6. Chromatograms from clinical urine samples.

3.6. Stability of MC and IS in plasma and urine extracts at 4 °C and in working solutions

MC and the IS were stable for up to 7 days in plasma and urine extracts, stored in the autosampler tray or in a refrigerator (Table 2). MC and the IS were stable in un-diluted extracts, containing 5% TCA, for at least 5 days (Table 2). MC was stable at ca. -80 °C in stock and working solutions stored at for up to 321 and 83 days respectively. The IS was stable in the intermediate, aqueous, working solution, at a concentration of 200 mg/L, for up to 279 days at ca. -80 °C.

3.7. Application to clinical samples

The robustness of the assays when applied to clinical samples is demonstrated in Table 6. For the purpose of ISRs, 99 of the 1423 plasma samples (6.96%) and 75 of the 689 urine samples (10.9%) were reanalysed. The results met the acceptance criteria that the difference between the original and repeat results should be <20%

Table 6
Performance of calibrators and QCs in application to a Phase 1 clinical study.

QC	Plasma				Urine			
	Accuracy of mean (%)	Precision, CV (%)	n	Failure rate (%)	Accuracy of mean (%)	Precision, CV (%)	n	Failure rate (%)
	Failed calibrators		1177	3.57	Failed calibrators		538	2.23
Low	106	9.30	116	13.8	102	8.60	50	6.00
Medium	107	7.69	118	11.9	104	3.43	52	1.92
High	104	5.61	118	2.54	96.5	3.89	51	3.92

n = number of replicates.

for >= 67% of ISRs. This also demonstrated the stability of MC and lack of significant epimerisation in both stored and reanalysed clinical samples (Figs. 5 and 6). The chromatographic resolution was particularly important for urine samples collected at later time points (Fig. 6) because of an abundance of 4-EMC.

4. Conclusion

Novel, uncomplicated, HPLC-MS/MS based assays for minocycline, a tetracycline antibiotic, in human plasma and urine have been developed and validated. An analogue internal standard, tetracycline, and a simple deproteinising step were used. The assays were successfully used to generate PK data from a Phase 1 clinical study. This is an example of modern analytical technology being used in support of a new application of an old drug that is still effective and widely used.

Conflict of interest

All authors declare no conflicts of interest.

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